Neisseria gonorrhoeae antibiotic resistance by Bio-Plex Analysis

Neisseria gonorrhoeae is the second most commonly reported sexually transmitted infection in the United States with an estimated 600,000 new infections each year as stated by the Centers for Disease Control and Prevention (CDC) (1). N. gonorrhoeae typically infects the urethra, cervix, rectum, pharynx, eyes, skin, and joints, which can cause painful inflammation and discharge. Asymptomatic, untreated, or undertreated infections can lead to disseminated gonococcal infections (a.k.a. arthritis-dermatitis syndrome) and a pelvic inflammatory disease resulting in preterm labor, pelvic pain, ectopic pregnancies, and sterility in women (1). Therefore, proper and successful antibiotic treatment for N. gonorrhoeae infections is very important. Unfortunately, N. gonorrhoeae has developed resistance to most of the available antibiotics, which include the sulfanilamides, penicillin, tetracycline, fluoroquinolones, macrolides, and spectinomycin (2). Within the past ten years, these multi-resistant strains have been developing resistance to the last recommended line of antibiotic therapy, the cephalosporins, in clinically significant numbers in Japan, Southeast Asia, Australia, and Hawaii (3-6). In 2008, these multi-resistant, cephalosporin non-susceptible N. gonorrhoeae isolates were isolated from patients in San Francisco, California (7). Therefore, it has become very important to provide antibiotic susceptibility profiles for N. gonorrhoeae isolates, a personalized medicine approach, to provide each patient with effective antibiotic treatment options (2, 8).

The Past and Present Status of Neisseria gonorrhoeae Antibiotic Resistance.

Ever since antibiotics were first used to treat N. gonorrhoeae in 1936, resistance has been rapidly developing under selective pressure due to the great ability of N. gonorrhoeae to rapidly develop chromosomal mutations and acquire mobile genetic elements from the environment. In 1936, sulfanilamides were used to treat N. gonorrhoeae infections, however, their efficacy was short-lived because of the rapid emergence of resistance due to chromosomal mutations resulting in methionine auxotrophy and the acquisition of plasmids that encode alternative target enzymes with low affinities for the antibiotic (2). In 1945, penicillin became the recommended antibiotic treatment for the next 44 years, with tetracycline as an alternative treatment option by the 1950’s. However, penicillin efficacy progressively declined initially due to a series of additive chromosomal mutations (penA, penB, penB/porB, and mtrR) and later by the acquisition and spread of plasmids that contain genes such as blaTEM, which encode a penicillinase first identified in the United States in 1976 (9). These chromosomal mutations occurred over time and with each new mutation, the effective drug concentration or minimum inhibitory concentration (MIC) increased concurrently to the level of full resistance. Subsequently, CDC treatment recommendations over the years incrementally increased the recommended dose of intramuscular procaine penicillin from 50,000 units in 1945 to 4.8 million units by the early 1970s (2).

In the early 1970’s to the early 1980’s, additive chromosomal mutations in three genes, mtrR, penB/porB, and rpsJ, resulted in low-level tetracycline resistance (10). In 1983 the first high-level tetracycline resistant N. gonorrhoeae clinical strain was isolated, which harbored a plasmid containing the tetM gene (11). Tetracycline resistance due to these additive chromosomal mutations and acquisition of plasmids was spreading to the extent that tetracycline was no longer a viable treatment option.

In 1985 due to the emerging penicillin resistance, ceftriaxone became a recommended regimen for the treatment of uncomplicated N. gonorrhoeae infections. By 1989, resistance to penicillin was sufficiently widespread that penicillin was no longer recommended. Ceftriaxone then became the recommended treatment for N. gonorrhoeae, with ciprofloxacin as an alternative treatment option.

In 1993, oral fluoroquinolones (ciprofloxacin, levofloxacin, and ofloxacin) and the oral third-generation cephalosporin, cefixime, were recommended as oral regimens for N. gonorrhoeae treatment. The emergence of quinolone-resistance due to chromosomal mutations in the gyrA and parC genes of N. gonorrhoeae was first identified in Hawaii and in Asia in 1991. By 2000, fluoroquinolone-resistant N. gonorrhoeae was increasingly observed in people who became infected in Asia, the Pacific Islands (including Hawaii), or California (12). In 2000, fluoroquinolones were no longer recommended in Hawaii. The elimination of fluoroquinolones from the recommended list of antibiotics used to treat N. gonorrhoeae continued at other sites throughout the United States when the number of fluoroquinolone-resistant isolates rose above 20%. In 2006, the CDC announced that fluoroquinolones are no longer recommended for treating N. gonorrhoeae infections in the United States (2).

Currently a single antibiotic class, the cephalosporins, is recommended for N. gonorrhoeae treatment. As stated by Workowski et al., “Ceftriaxone, available only as an injection (125 mg intramuscularly), is the recommended regimen for uncomplicated urogenital and anorectal infection. Cefixime, 400 mg, is the only oral regimen recommended for gonorrhea treatment. Because cephalosporins are the only currently recommended class of antimicrobials, it is critical that susceptibility to these drugs be actively monitored. Recent reports from Japan and several other countries in Western Pacific Region suggest decreased susceptibility to cephalosporins, with the reported mechanism of resistance being alterations in penA genes. Additionally, mutations in mtrR, penA, and porB also contribute to cephalosporin non-susceptibility (13). Some N. gonorrhoeae strains demonstrating reduced cephalosporin susceptibility also have reduced susceptibility to multiple drug classes, including quinolones, macrolides, penicillins, and tetracyclines. The emergence and dissemination of such strains is of particular concern” (2).

A recent review provides a bleak picture of the current state of N. gonorrhoeae antibiotic resistance: “In Japan, N. gonorrhoeae is
resistant to penicillins, fluoroquinolones, tetracyclines and macrolides in the rate of 100%, 70%, 60%, 80%, respectively. In addition to penicillin-, macrolide-, tetracycline- and fluoroquinolone resistance, Neisseria gonorrhoeae acquired resistance to almost all of the oral and parenteral cephalosporins except for ceftriaxone and cefodizime, and was named CZRNG. The incidence rate of CZRNG is now 40% and such strains are simultaneously resistant to fluoroquinolones, tetracyclines, and macrolides. Therefore, 40% of N. gonorrhoeae strains are considered to be so-called multi-drug-resistant and this type of resistant strains was distributed in various parts of Japan. Due to that, fluoroquinolones and cefixime are not recommended for the treatment of gonococcal infection in Japan. The only therapeutic option left includes intramuscular/intravenous ceftriaxone. Although there is no study comparing 125 mg intramuscular and 1.0 g intravenous dose of ceftriaxone, 1.0 g intravenous dose of ceftriaxone is recommended rather than 125 mg intramuscular regimen for the treatment of gonococcal infection in Japan, because resistance of N. gonorrhoeae to ceftriaxone has been gradually shifting to higher MIC levels." (4). Furthermore, the spread of fluoroquinolone resistant and cephalosporin non-susceptible N. gonorrhoeae isolates followed a similar path to the United States. These multi-drug resistant isolates were first identified in Japan and Southeast Asia, followed by Australia and the Pacific Islands, ultimately spreading to San Francisco, CA. Fluoroquinolone resistant isolates have now populated major cities across the United States. Therefore, it may only be a matter of time until the last current line of antibiotic therapy for gonorrhea, the cephalosporins, are not recommended for therapy due to high levels of resistance.

Alternative therapies are available although not currently recommended for gonorrhea by the CDC. These include two macrolides, azithromycin and erythromycin, doxycycline and the aminoglycoside spectinomycin. Azithromycin or doxycycline, and ceftriaxone are used in combination therapy for N. gonorrhoeae and Chlamydia trachomatis co-infections. However, resistance mechanisms for these antibiotics have been discovered in N. gonorrhoeae. Macrolide resistance is associated with mtrR mutations, the acquisition of a mef efflux pump, and 23S rRNA rrf mutations C2599T (moderate-level resistance), and A2143G (high-level resistance) (14). Plasmid and chromosomal mechanisms of tetracycline resistance can confer cross-resistance to doxycycline (15). Spectinomycin resistance was found to be due to 16S rRNA G1064C and C1192U mutations (16).

After particular antibiotics were discontinued, the antibiotic resistance profiles of N. gonorrhoeae changed. The introduction of penicillin treatment was followed by a reduction of sulphonamide-resistant N. gonorrhoeae from a peak of 85% in 1949-1950 to 5% of strains examined ten years later (17). Recent tests of the nutritional requirements of gonococcal strains isolated over a period of 40 years revealed that methionine auxotrophy was very common in the sulphonamide era, but had mostly disappeared by 1970 (18). In 1998, 29.4% of the clinical gonococcal isolates collected throughout the U.S. by the CDC Gonococcal Isolate Surveillance Project were found to be resistant to either penicillin, tetracycline, or both. Interestingly, the percentage of penicillinase-producing N. gonorrhoeae organisms declined significantly during the years 1991 to 1998, while the percentage of chromosomally-mediated resistant N. gonorrhoeae isolates increased (19). This observation may also be in conjunction with the use of fluoroquinolones, which was first recommended in 1993. It was found that fluoroquinolone use caused a decline in the prevalence of penicillinase and tetM conjugative plasmids (20). Also, some of the same chromosomal mutations that lead to penicillin resistance are also responsible for cefalosporin non-susceptibility.

The Need for Neisseria gonorrhoeae Antibiotic Susceptibility Surveillance

In light of the ever-changing landscape of N. gonorrhoeae antibiotic susceptibility, a personalized medicine approach is necessary. The use of molecular diagnostics such as PCR can provide a full susceptibility profile of individual N. gonorrhoeae isolates. As stated by a leader in the N. gonorrhoeae field, Dr. John Tapsall, “It is possible that enhancements or modifications to nucleic acid amplification assays will also allow determination of antimicrobial resistance patterns in the medium term. Such a development would facilitate assessment of antimicrobial resistance patterns and would greatly enhance the ability to recommend appropriate treatments” (21). With the personalized medicine approach of determining the full susceptibility profile, the physician can make an educated choice of which antibiotics to use. Even though the antibiotics used in the past, i.e. penicillin, tetracycline, and fluoroquinolones, are not currently recommended for N. gonorrhoeae treatment, physicians can choose those antibiotics when demonstrated that the bacterial isolate is susceptible. Using older antibiotics and not overusing the last currently recommended cephalosporin drug class, specifically ceftriaxone, can extend the life of the cephalosporins and slow down the development of resistance. History has shown that indiscriminate overuse of a single antibiotic will increase the selective pressure and subsequently lead to an increase in antibiotic resistance. With no alternative antibiotics in development for N. gonorrhoeae presently or expected to be available in the near future, having the option of multiple antibiotic classes is an effective method of N. gonorrhoeae therapy.

Neisseria gonorrhoeae antibiotic resistance by Bio-Plex Analysis

The Neisseria gonorrhoeae antibiotic resistance by Bio-Plex Analysis assay provides a simple, non-invasive method of determining gonorrhea infections and assessment of N. gonorrhoeae specific genetic markers of antibiotic resistance from a sample collected via the OneSwab® specimen collection platform. This assay does not involve the isolation of live bacterial cells from the OneSwab® specimen. Instead, it screens for N. gonorrhoeae specific genes and mutations from DNA extracted from the OneSwab® collection system.

While susceptibility testing by culture methods remains a gold standard for antibiotic susceptibility determination in clinical microbiology, contemporary molecular nucleic acid based methods can also reliably detect genetic determinants of antibiotic resistance. Known mechanisms of antibiotic resistance in N. gonorrhoeae are linked to mutations in the chromosomal DNA as well as the presence of plasmid-borne genes. Surveillance of genetic markers of antibiotic resistance is important for prediction of clinical resistance.

Due to the genome plasticity of N. gonorrhoeae strains circulating in the population, this bacteria has developed resistance to multiple classes of antimicrobial agents resulting in decreased efficacy for gonorrhea therapy. Antibiotic susceptibility signatures of individual N. gonorrhoeae strains differ from each other. The Neisseria gonorrhoeae antibiotic resistance by Bio-Plex Analysis assay developed by Medical Diagnostic Laboratories, L.L.C. (MDL) provides a valuable diagnostic tool for the prediction of antibiotic susceptibility of individual N. gonorrhoeae strains in a given clinical specimen. This test addresses the problem of genetic variability in N. gonorrhoeae and delivers a prognostic recommendation for antibiotic therapy in a personalized manner.
The test screens for \textit{N. gonorrhoeae} genes and mutations known to confer resistance to the major antimicrobials used for gonorrhea infection treatment: cefixime, penicillin, ciprofloxacin, tetracycline, azithromycin, and spectinomycin (Table 1).

Table 1: Antimicrobial resistance tested for in Test 167 \textit{Neisseria gonorrhoeae} antibiotic resistance by Bio-Plex Analysis assay

<table>
<thead>
<tr>
<th>Antimicrobials</th>
<th>Mutations / Marker</th>
<th>Resistance in \textit{Neisseria gonorrhoeae}</th>
<th>Assay References</th>
</tr>
</thead>
<tbody>
<tr>
<td>cefixime</td>
<td>Asp345A, G542S, G545S, P551S and P551L</td>
<td>Mutations in penA gene can confer resistance to penicillin and cefixime, and reduced susceptibility to ceftriaxone.</td>
<td>(22, 27, 24, 25)</td>
</tr>
<tr>
<td>penicillin</td>
<td>S91P, D95A, D95G, S91L, S91F, D95Y</td>
<td>Mutations in gyrA gene can confer resistance to fluoroquinolones (ciprofloxacin).</td>
<td>(26, 27, 28, 29)</td>
</tr>
<tr>
<td>tetracycline</td>
<td>L421P</td>
<td>Mutations in penA gene can confer resistance to penicillin.</td>
<td>(32, 33)</td>
</tr>
<tr>
<td>azithromycin</td>
<td>G120D, G120K, A121D, A121S, A121G</td>
<td>Mutations in penB (porB) gene can confer resistance to penicillin and tetracycline.</td>
<td>(10, 34, 35)</td>
</tr>
<tr>
<td>spectinomycin</td>
<td>G45D, G45S, -35delA, -10insTT</td>
<td>Mutations in mtrR gene can confer resistance to penicillin, fluoroquinolones (ciprofloxacin), tetracycline, and azithromycin.</td>
<td>(36, 37, 38, 39, 40, 47)</td>
</tr>
<tr>
<td>tetM</td>
<td>tetM</td>
<td>Presence of tetM gene can confer resistance to tetracycline.</td>
<td>(38, 41, 42, 43)</td>
</tr>
<tr>
<td>rpsJ</td>
<td>V57M</td>
<td>Mutations in rpsJ gene can confer resistance to tetracycline.</td>
<td>(10, 38)</td>
</tr>
<tr>
<td>bla</td>
<td>bla\textsubscript{TEM-1}</td>
<td>Presence of bla gene can confer resistance to penicillin.</td>
<td>(9, 44, 45)</td>
</tr>
<tr>
<td>16s rRNA</td>
<td>G1064C, C1192U</td>
<td>Mutations in 16s rRNA gene can confer resistance to spectinomycin.</td>
<td>(16)</td>
</tr>
</tbody>
</table>

The association between the presence of particular genetic markers and antimicrobial resistance of \textit{N. gonorrhoeae} has been confirmed by multiple studies (Table 2). Since our understanding of the molecular mechanisms of antimicrobial resistance continues to expand, in the future the assay can be augmented by incorporation of newly discovered markers of antibiotic resistance making it even more thorough and continually comprehensive.

The new MDL \textit{Neisseria gonorrhoeae} antibiotic resistance by Bio-Plex Analysis assay screens for 30 genetic markers of antibiotic-resistance in 10 \textit{N. gonorrhoeae} genes (28 point mutations in 8 chromosomal genes and 2 plasmid genes) and 1 \textit{N. gonorrhoeae} species-specific marker (Table 2). The assay employs multiplex PCR amplifying 12 loci in the \textit{N. gonorrhoeae} genome followed by a multiplexed allele-specific primer extension reaction targeting specific antibiotic resistance mutations and genes. Detection and sorting of extended allele-specific primers are achieved through a Bio-Plex liquid microarray analysis utilizing beads that are both tagged and fluorescently labeled (Figure 1). This allows for the analysis of multiple genetic markers from a single specimen in a single reaction in a manner that is accurate, sensitive and highly specific.

![Assay Procedural Layout](image)

Figure 1. Assay Procedural Layout.

Table 2. Genetic Markers included in Test 167 \textit{Neisseria gonorrhoeae} antibiotic resistance by Bio-Plex Analysis assay

Assay References
